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# Flavonoids Suppress *Pseudomonas aeruginosa* Virulence through Allosteric Inhibition of Quorum-sensing Receptors*[S]

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**Quorum sensing is a process of cell-cell communication that bacteria use to regulate collective behaviors. Quorum sensing depends on the production, detection, and group-wide response to extracellular signal molecules called autoinducers. In many bacterial species, quorum sensing controls virulence factor production. Thus, disrupting quorum sensing is considered a promising strategy to combat bacterial pathogenicity. Several members of a family of naturally produced plant metabolites called flavonoids inhibit *Pseudomonas aeruginosa* biofilm formation by an unknown mechanism. Here, we explore this family of molecules further, and we demonstrate that flavonoids specifically inhibit quorum sensing via antagonism of the autoinducer-binding receptors, LasR and RhlR. Structure-activity relationship analyses demonstrate that the presence of two hydroxyl moieties in the flavone A-ring backbone are essential for potent inhibition of LasR/RhlR. Biochemical analyses reveal that the flavonoids function non-competitively to prevent LasR/RhlR DNA binding. Administration of the flavonoids to *P. aeruginosa* alters transcription of quorum sensing-controlled target promoters and suppresses virulence factor production, confirming their potential as anti-infectives that do not function by traditional bacteriocidal or bacteriostatic mechanisms.**

Quorum sensing (QS)[2] is a bacterial cell-cell communication process that controls collective behaviors (1). QS relies on the production, accumulation, detection, and population-wide response to extracellular signaling molecules called autoinducers (AIs) (2). The *Pseudomonas aeruginosa* QS circuit consists of two primary AI synthase/receptor pairs, LasI/R and RhlI/R, which produce and detect $3OC_{12}$-homoserine lactone ($3OC_{12}HSL$) and $C_4$-homoserine lactone ($C_4HSL$), respectively (3–6). At high cell density, LasR and RhlR, which are members of the large family of LuxR-type proteins, bind their cognate AIs, dimerize, bind DNA, and activate expression of genes encoding functions required for virulence and biofilm formation as well as other processes not involved in pathogenicity (7).

*P. aeruginosa* is a pathogen of clinical relevance that affects cystic fibrosis sufferers, burn victims, immunocompromised individuals, and patients with implanted medical devices, such as intubation tubes (8, 9). *P. aeruginosa* frequently forms biofilms on medical surfaces, leading to nosocomial infections. *P. aeruginosa* has acquired resistance to commonly used antibiotics and is now a priority pathogen on the Centers for Disease Control and Prevention ESKAPE pathogen list (10, 11). New anti-infective approaches are urgently needed for *P. aeruginosa*, and targeting bacterial behaviors, such as QS, rather than targeting bacterial growth, represents an attractive alternative for exploration in anti-microbial research (1, 12). Such therapies could minimize selection for drug resistance, potentially endowing these medicines with extended functional lifetimes.

Previous efforts to develop *P. aeruginosa* QS inhibitors include screening of natural products, screening of small molecule libraries, *in silico* screening, and synthesis of focused libraries based on the native AI structures (13–16). These efforts resulted in the discovery of several competitive LasR inhibitors that function *in vitro* but not *in vivo* in an animal infection model (17). However, one *P. aeruginosa* QS inhibitor, *meta*-bromothiolactone (mBTL), discovered through synthesis of focused libraries, inhibits QS both *in vitro* and *in vivo* in a *Caenorhabditis elegans* model of infection; mBTL inhibits LasR and RhlR via competition with the natural AIs for occupancy of the ligand binding sites (18).

Flavonoids are a group of natural products that exhibit broad pharmacological activities ranging from anti-microbial to anti-inflammatory (19). Recently, multiple flavonoids were reported to inhibit *P. aeruginosa* biofilm formation, raising the possibility that they function by affecting QS signaling (20–22). How-

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[S] This article contains supplemental Table 1 and Figs. 1–3.

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[2] The abbreviations used are: QS, quorum sensing; AI, autoinducer; CL, chlorolactone; CRISPR, clustered regularly interspaced short palindromic repeats; DPD, 4,5-dihydroxy-2,3-pentanedione; HSL, homoserine lactone; LBD, ligand-binding domain; mBTL, *meta*-bromothiolactone; PA14, *P. aeruginosa* UCBPP-PA14; RLU, relative light units; SAR, structure-activity relationship; mNG, mNeonGreen; IPTG, isopropyl 1-thio-β-D-galactopyranoside; ANOVA, analysis of variance.

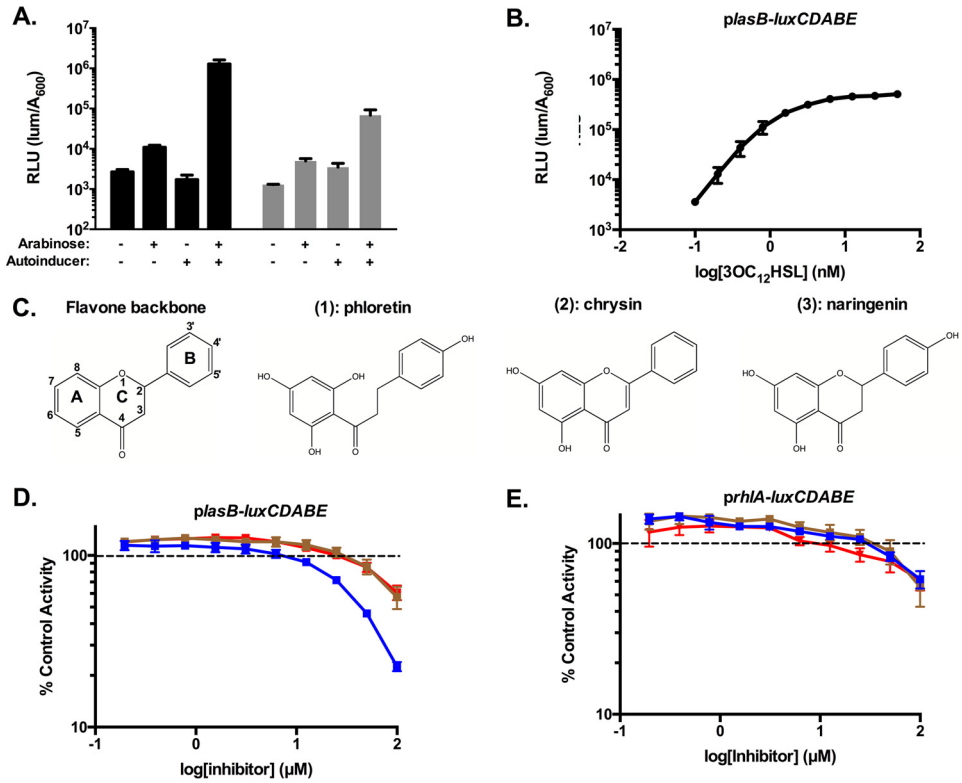


FIGURE 1. **A high throughput screen reveals flavonoids as inhibitors of LasR and RhlR.** *A*, *E. coli* reporter strains harboring arabinose-inducible *lasR* and a plasmid containing p*lasB-luxCDABE* (*black*, called the LasR reporter strain) or arabinose-inducible *rhlR* and p*rhlA-luxCDABE* (*gray*, called the RhlR reporter strain). 0.1% (v/v) arabinose, 100 $\mu$M $3OC_{12}HSL$ (LasR experiments), and 100 $\mu$M $C_4HSL$ (RhlR experiments) were provided as designated. RLU are defined as light production (absorbance units (*lum*)) divided by $A_{600}$. *B*, response of the LasR reporter strain to different concentrations of $3OC_{12}HSL$ in the presence of 0.1% arabinose. The $EC_{75}$ for $3OC_{12}HSL$ is 2.5 nM, which is the concentration used for screening. *C*, flavonoid nomenclature and the A-, B-, and C-rings are shown for the backbone molecule as well as the three flavonoid compounds identified in the high throughput screen. *D*, response of the LasR reporter strain to phloretin (**1**) (*blue*), chrysin (**2**) (*brown*), and narigenin (**3**) (*red*) in the presence of 2.5 nM $3OC_{12}HSL$ and 0.1% arabinose. % *Control Activity*, data normalized to data obtained in the absence of inhibitor compound ($n = 3$). The *dotted line* shows 100%. *E*, as in *D* using the RhlR reporter stain and 10 $\mu$M $C_4HSL$. *Error bars*, S.E.

ever, their mechanisms of action were not investigated. Here, we show that novel flavonoids possessing dihydroxyl moieties in the flavone A-ring backbone, as well as the previously identified flavonoids baicalein and quercetin, bind to the QS receptors, LasR and RhlR, and significantly reduce their ability to bind to DNA encoding QS-regulated promoters. Structure-activity relationship (SAR) analyses indicate that the presence of two hydroxyl groups in the flavone A-ring is necessary for inhibition of LasR and RhlR. Using LasR as the representative receptor, we show that the flavonoids act by an allosteric mechanism. The flavonoids inhibit virulence factor production and swarming in a LasR/RhlR-dependent manner. These compounds are the first noncompetitive QS inhibitors identified that target LasR/RhlR and prevent DNA binding. Halogenated furanones have been discovered that function non-competitively by destabilizing LasR, promoting its degradation (23–25). Many flavonoids are GRAS (generally recognized as safe) compounds and thus could immediately be explored for uses in industry, agriculture, and animal husbandry. Our results support the general notion that targeting QS represents a viable route for controlling *P. aeruginosa* pathogenicity. Presumably, strategies analogous to those presented here could be used to control other pathogens that use QS to regulate virulence, biofilm formation, or other traits for which inhibition on demand would be useful (26).

## Results

*Discovery of Flavonoid LasR and RhlR Inhibitors*—To screen for and characterize *P. aeruginosa* QS inhibitors, we constructed an *Escherichia coli* strain harboring arabinose-inducible LasR or RhlR and a LasR- or RhlR-controlled promoter fused to luciferase (*lasB-luxCDABE* for LasR and *rhlA-luxCDABE* for RhlR). In the presence of arabinose and AI ($3OC_{12}HSL$ for *E. coli* carrying LasR and *lasB-luxCDABE* and $C_4HSL$ for *E. coli* carrying RhlR and *rhlA-luxCDABE*), the *E. coli* reporter strains produce high levels of light. Only low level, residual light is produced if nothing, only AI, or only arabinose is supplied (Fig. 1*A*).

We used the *E. coli* strain carrying LasR and *lasB-luxCDABE* to identify putative QS inhibitors. We screened 60,000 molecules from a high diversity chemical library for those compounds that reduced bioluminescence emission by 3 S.D. values relative to the control sample. We supplied the native AI, $3OC_{12}HSL$, at its $EC_{75}$ (2.5 nM) (Fig. 1*B*) and set a $Z$ score of $<-3$ from plates with a $Z'$ score of $>0.7$ as criteria for hit identification. Both bioluminescence and $A_{600}$ were measured, the latter to eliminate molecules that decreased light production by inhibiting growth. We performed a secondary control screen with an *E. coli* strain carrying LasR and a constitutive promoter fused to *luxCDABE* to exclude molecules that inhib-

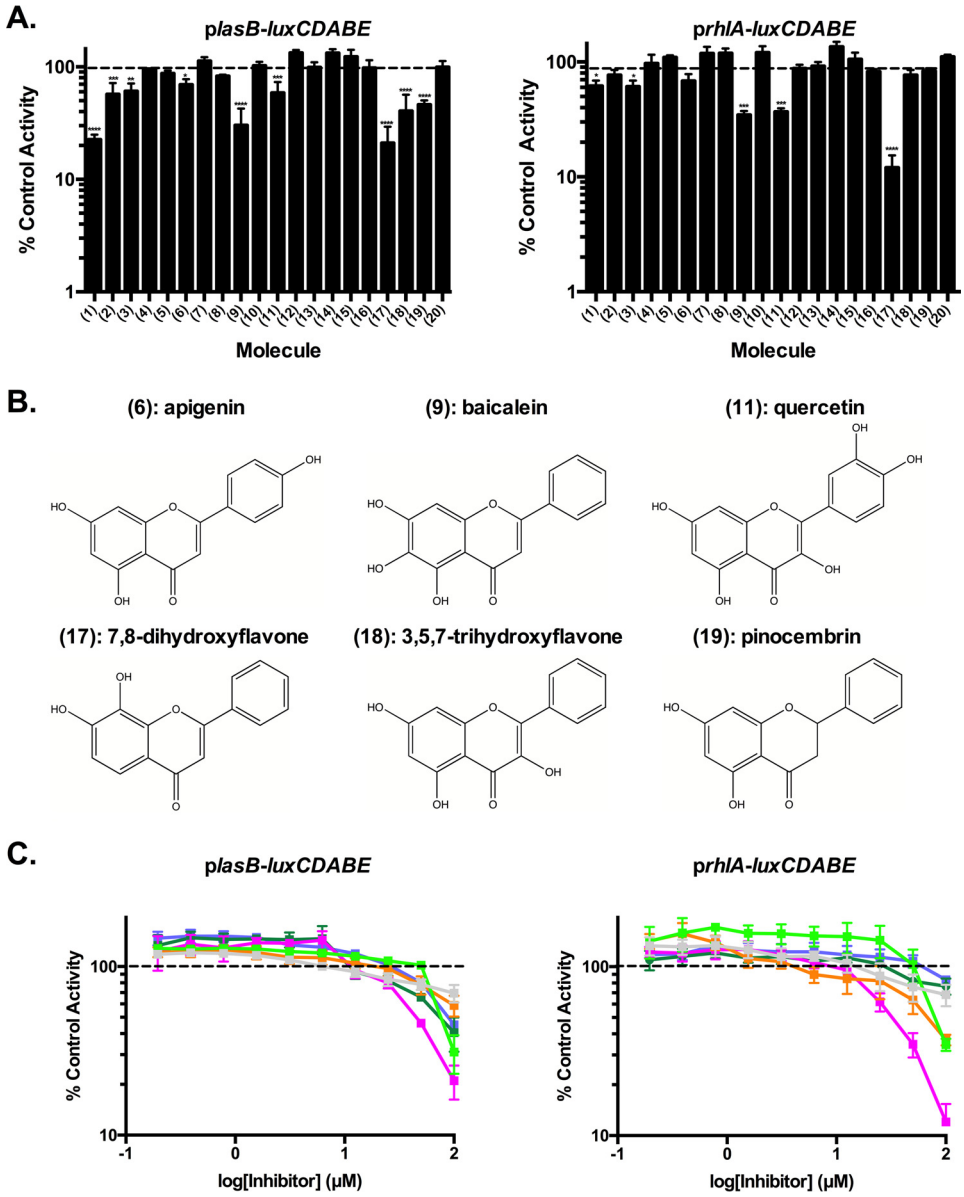


FIGURE 2. **Structure-activity relationship for flavonoid inhibition of LasR and RhlR.** *A*, all flavonoid compounds in supplemental Table 1 were tested at 100 $\mu$M for inhibition in the LasR reporter strain in the presence of 2.5 nM $3OC_{12}$HSL and 0.1% arabinose (*left*) and for inhibition of the RhlR reporter strain in the presence of 10 $\mu$M $C_4$HSL and 0.1% arabinose (*right*). Percentage of control activity was calculated for each molecule ($n = 3$). The *dotted lines* indicate 100%. Statistical significance, relative to the DMSO control, was determined using an ANOVA test with Tukey-Kramer post hoc analysis. ****, $p < 0.0001$. Structures (*B*) and concentration-dependent response inhibition curves (*C*) of flavonoids apigenin (**6**) (*silver*), baicalein (**9**) (*light green*), quercetin (**11**) (*orange*), 7,8-dihydroxyflavone (**17**) (*magenta*), 3,5,7-trihydroxyflavone (**18**) (*dark green*), and pinocembrin (**19**) (*purple*) in the LasR (*left*) and RhlR (*right*) reporter assays. LasR reporter assays included 2.5 nM $3OC_{12}$HSL, and RhlR reporter assays included 10 $\mu$M $C_4$HSL. In both cases, arabinose was added at 0.1%. *Error bars*, S.E.

ited luciferase activity. We obtained 32 hit molecules for follow-up analysis.

The set of LasR hit compounds contained three flavonoids, phloretin (**1**), chrysin (**2**), and naringenin (**3**) (Fig. 1, *C* and *D*). All three compounds also reduced light production in the *E. coli* reporter strain containing RhlR and *rhlA-luxCDABE* (Fig. 1*E*) but displayed no activity in the constitutive light-producing reporter strain (supplemental Fig. 1). Together, these results suggest that these flavonoids are dual LasR/RhlR inhibitors.

*SAR of Flavonoid Inhibition of LasR and RhlR*—All three flavonoid compounds identified above contain hydroxyl groups in the 5- and 7-positions of the A-ring (Fig. 1*C*). Additionally, phloretin (**1**) does not contain the conformational constraint of the chromanone/chromenone C-ring present in chrysin (**2**) and naringenin (**3**). To ascertain the importance of the hydroxyl moieties in the A- and B-ring and the contribution of the C-ring to inhibition of LasR and RhlR, we conducted a focused structure-activity study. We tested 20 structural analogs at high concentration (100 $\mu$M) against each receptor (Fig. 2*A*). Six flavonoid compounds (Fig. 2*B*), in addition to the three discovered in the screen, exhibited inhibition and were subsequently examined in 10-point antagonist dose-response assays against each receptor to define potency and efficacy (Fig. 2*C* and supplemental Table 1). All nine compounds inhibited both receptors in dose-dependent manners. We do note that at low con-

centrations, and only in the presence of AIs, the flavonoids modestly activate the reporter strains (Figs. 1*D* and 2*C*). This feature will need to be considered if these compounds are further explored for applications.

*The A-ring SAR*—We first performed a systematic survey of the A-ring hydroxyl groups to determine their importance in LasR/RhlR inhibition. The three monohydroxy flavones, 5-hydroxyflavone (**7**), 6-hydroxyflavone (**13**), 7-hydroxyflavone (**8**), and the flavonoid lacking any A-ring hydroxyl group, flavone (**4**), were all inactive (Fig. 2 (*A* and *B*) and supplemental Table 1). Of the dihydroxy analogs tested, chrysin (**2**) and 7,8-dihydroxyflavone (**17**) are potent LasR/RhlR inhibitors, whereas 5,6-dihydroxyflavone (**16**) displayed no activity. The corresponding 6,8-dihydroxyflavone was not available for testing. These data suggest that 1) two hydroxyl groups are necessary for potent inhibition and 2) one of them must be at position 7 on the A-ring. The presence of three hydroxyl groups on the A-ring (*i.e.* baicalein (**9**)) is tolerated but does not increase inhibitory potency. A free hydroxyl group is essential for activity, because the methyl ether analogs 5,7-dimethoxyflavone (**12**), 5,7,4′-trimethoxyflavone (**14**), and 5,7-dimethoxy-4′-hydroxyflavone (**15**) were all inactive. Thus, either the binding pocket that the A-ring fits into is small or the presence of hydrogen bond donors on the A-ring is important for activity.

*The C-ring SAR*—None of the modifications that we examined in the C-ring appeared to significantly influence LasR/RhlR inhibition. Specifically, the double bond between positions 2 and 3 is not a strict requirement for activity because naringenin (**3**) and pinocembrin (**19**) lack the C-ring double bond and possess inhibitory capability (Fig. 2 (*A* and *B*) and supplemental Table 1). The presence of a C-3 hydroxyl moiety is tolerated, because quercetin (**11**) and 3,5,7-trihydroxyflavone (**18**) are LasR/RhlR inhibitors (Fig. 2 (*A* and *B*) and supplemental Table 1). Finally, as noted above, phloretin (**1**) does not contain the C-ring chromanone structure yet retains activity. This result suggests that it is able to adopt a conformation similar to that of the chromanone/chromenone-based flavonoids when binding to LasR/RhlR.

*The B-ring SAR*—Hydroxyl groups on the B-ring are not absolutely required for LasR/RhlR inhibitory activity because a number of analogs with a simple phenyl B-ring are active (e.g. quercetin (**11**)). The presence of 3′- and 4′-hydroxyl groups in the B-ring is tolerated, but these groups do not enhance LasR/RhlR inhibition. The presence of methyl ether groups, by contrast (acacetin (**5**) and diosmetin (**10**)), eliminated inhibitory activity, which suggests a tight steric constraint within the protein binding pocket that the B-ring occupies (Fig. 2 (*A* and *B*) and supplemental Table 1).

Taken together, our data reveal that, with respect to LasR/RhlR inhibition by the representative set of flavonoids tested here, there is a requirement for a hydroxyl group at position 7 of the A-ring combined with at least one other hydroxyl group elsewhere on the A-ring. Additional hydroxyl groups in the A-ring are tolerated, whereas larger methyl ethers are not. The C- and B-rings can accommodate many substitutions, with the exception that methyl groups on the B-ring are not tolerated. Some of the flavonoids that inhibit LasR are also capable of inhibiting RhlR (*e.g.* 7,8-dihydroxyflavone (**17**)), whereas some are not (*e.g.* pinocembrin (**19**); Fig. 2 (*A* and *B*) and supplemental Table 1).

*Dihydroxyl Flavonoids Show Cross-species Receptor-inhibitory Activity*—We wondered whether flavonoid inhibition was restricted to the *P. aeruginosa* LasR and RhlR receptors or whether flavonoids generally inhibit AI binding QS receptors. To explore specificity, we examined several flavonoids for inhibition of another LuxR type protein, CviR, which, when bound to its cognate AI, $C_6$HSL, activates expression of the *vioA* promoter. We measured a *vioA-gfp* transcriptional fusion as the readout (Fig. 3*A*). As a control, we used $C_{10}$HSL, which is a competitive inhibitor of $C_6$HSL (27). None of the flavonoids significantly inhibited CviR function (Fig. 3*A*). Conversely, $C_6$HSL could also not activate LasR in our reporter assay (Fig. 3*B*). Thus, there appears to be no cross-activation and no cross-inhibition between these receptors at least with respect to the molecules under study here. We also examined the flavonoids for inhibition of LuxN, the *Vibrio harveyi* AI receptor that detects the AI 3OHC$_4$HSL (28). Unlike LasR, RhlR, CviR, and other LuxR-type receptors, LuxN is a transmembrane protein that transduces the AI binding event into the cell via a phosphorylation-dephosphorylation cascade. In *V. harveyi*, bioluminescence is the endogenous output of QS signal transduction. Specifically, the AI, 3OHC$_4$HSL, stimulates light production, and this activity depends on LuxN. Phloretin (**1**) and quercetin (**11**) inhibited LuxN-dependent light production, although less potently than does the previously characterized inhibitor, chlorolactone (CL) (Fig. 3*C*) (28, 29). Thus, both types of antagonists that we have discovered, structural analogs of HSL AIs and the structurally distinct flavonoid analogs, inhibit LuxN. By contrast, CviR and LasR/RhlR show specificity with respect to antagonism, in agreement with previous findings that CL specifically inhibited CviR but not RhlR and LasR (18). These results indicate that flavonoids could conceivably be used to target QS networks of multiple bacterial species. However, their ability to do so must be evaluated on a case-by-case basis because their SARs may differ depending on receptor type.

*Flavonoids Inhibit LasR and RhlR through a Non-competitive Mechanism*—The discovery of flavonoids as a new class of LasR/RhlR inhibitors is intriguing because they are not structurally similar to the native AIs or to previously reported competitive LasR/RhlR inhibitors, which are all structural analogs of the AIs (13, 14, 30). It is possible that the flavonoids function competitively by occupying the ligand-binding site. We call this mechanism 1. Other potential mechanisms of inhibition are equally plausible and include inhibition of receptor stability/solubility (mechanism 2), disruption of receptor dimerization (mechanism 3); impairment of DNA binding (mechanism 4); and interference with RNA polymerase engagement (mechanism 5). Here, using LasR as the representative receptor, we examine possible mechanisms to characterize how the flavonoid inhibitors function.

We first tested whether the flavonoids are competitive inhibitors of LasR (mechanism 1). We assayed the *E. coli* bioluminescent reporter strain for inhibition of light production by 100 $\mu$M flavonoid in the presence of different concentrations of the native AI, 3OC$_{12}$HSL. If the inhibition mechanism is competi-

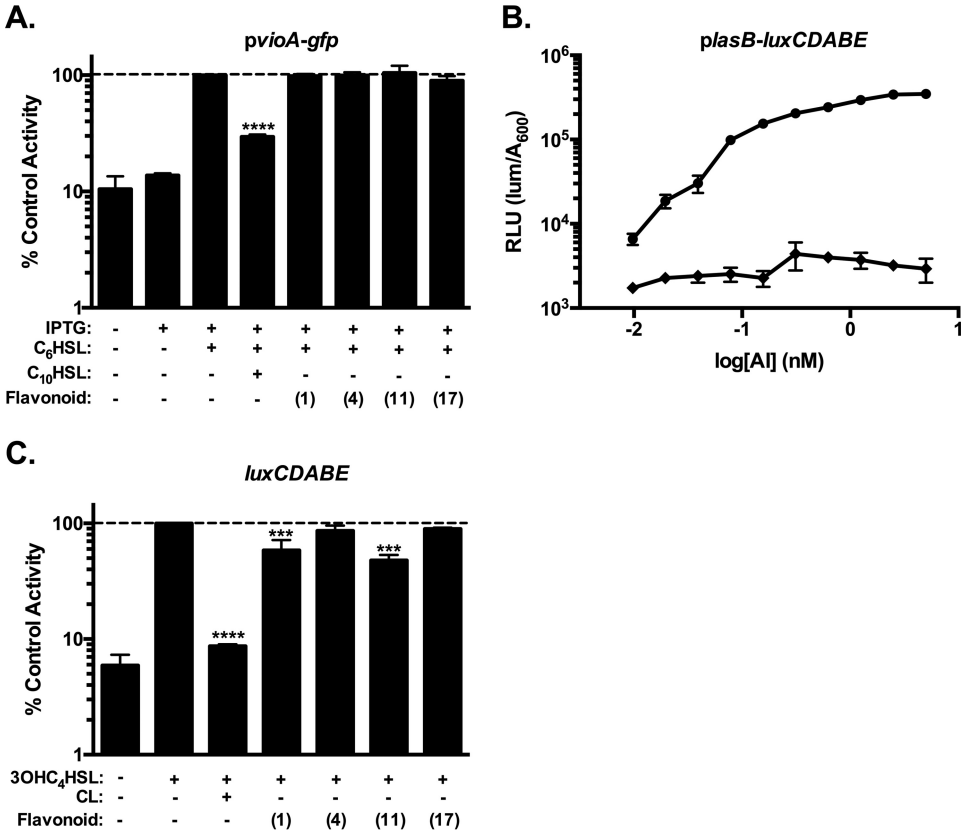


FIGURE 3. **Flavonoids inhibit the *V. harveyi* LuxN receptor but not the *C. violaceium* CviR receptor.** *A*, flavonoid inhibition was tested in an *E. coli* CviR reporter strain harboring IPTG-inducible CviR and p*vioA-gfp*. 1 mM IPTG and 100 $\mu$M $C_6$HSL, $C_{10}$HSL, and/or flavonoids were provided as indicated. Percentage of control activity represents data normalized to the no inhibitor control (*dotted line*) ($n = 3$). *B*, the LasR reporter strain in response to $3OC_{12}$HSL (*circles*) and $C_6$HSL (*diamonds*) at the designated concentrations. *C*, flavonoid inhibition of a bioluminescent *V. harveyi* LuxN reporter strain. 20 nM $3OHC_4$HSL and a 100 $\mu$M concentration of the flavonoids were provided as indicated. CL, the known LuxN inhibitor, was provided at 100 $\mu$M. Percentage of control activity represents data normalized to the no addition control (*dotted line*) ($n = 3$). *Error bars*, S.E. In *A* and *C*, statistical significance was determined using an ANOVA test with Tukey-Kramer post hoc analysis. In *A*, the comparison is with the IPTG + $C_6$HSL control, and in *C*, the comparison is with the DMSO control. ****, $p < 0.0001$; ***, $p < 0.001$.

tive, the expectation is that for each 10-fold increase in AI concentration assayed, a corresponding 10-fold decrease in inhibitor potency should occur. However, Fig. 4*A* shows that phloretin (**1**), chrysin (**2**), baicalein (**9**), quercetin (**11**), and 7,8-dihydroxyflavone (**17**) inhibited light production in a manner that was independent of AI concentration. These results suggest that the flavonoid inhibitors do not act via competition with the AI for the ligand-binding pocket.

LasR and related proteins, including RhlR, do not fold and are thus insoluble in the absence of their cognate AI ligands (31–35). Exogenously supplied AIs, synthetic agonists, or antagonists that bind in the ligand-binding pockets typically allow LasR-type proteins to fold and therefore become soluble. We reasoned that if the addition of flavonoids stabilized LasR in a soluble form, it would indicate that flavonoids functioned by a competitive inhibition mechanism. If not, it would further confirm the results in Fig. 4*A* indicating that the flavonoids are not competitive inhibitors. In the absence of any ligand, when LasR is overexpressed in *E. coli*, no LasR can be detected in the soluble fraction (Fig. 4*B*). As expected, administration of exogenous $3OC_{12}$HSL at 100 $\mu$M caused a significant fraction of the LasR protein to become soluble. Similarly, mBTL, which binds in the ligand binding site, also solubilizes LasR (Fig. 4*B*). By contrast, the addition of chrysin (**2**) or 7,8-dihydroxyflavone (**17**) did not cause LasR to be stabilized (Fig. 4*B*). These results indicate that flavonoids do not bind in the ligand binding pocket in LasR, validating the data in Fig. 4*A*. We conclude that flavonoids do not function competitively (*i.e.* they do not function by mechanism 1 above). Of note, the flavonoids also do not decrease LasR solubility in the presence of $3OC_{12}$HSL (Fig. 4*B*), showing that they also do not function by mechanism 2 above. Together, the dose-response and solubility analyses eliminate mechanism 1 (competitive inhibition) and mechanism 2 (inhibition of receptor stability/solubility).

*Flavonoids Prevent LasR from Binding DNA*—We next explored the possibility that the flavonoids function by mechanism 3, disruption of LasR dimerization (36). To do this, we expressed and purified full-length LasR bound to $3OC_{12}$HSL in the presence and absence of 100 $\mu$M quercetin (**11**) or 7,8-dihydroxyflavone (**17**). Size exclusion gel filtration showed that there was no significant difference between the protein preparations (Fig. 5*A*). In all cases, the predominant form of LasR eluted as a dimer. Thus, the flavonoids do not inhibit LasR by mechanism 3, disruption of dimer formation.

To test mechanism 4, impairment of DNA binding, we employed electrophoretic mobility shift assays to assess LasR-$3OC_{12}$HSL binding to radiolabeled *lasB* promoter DNA in the presence and absence of flavonoids. Importantly, the *lasB* promoter DNA sequence that we used in the mobility shift analyses is identical to that cloned upstream of the luciferase reporter

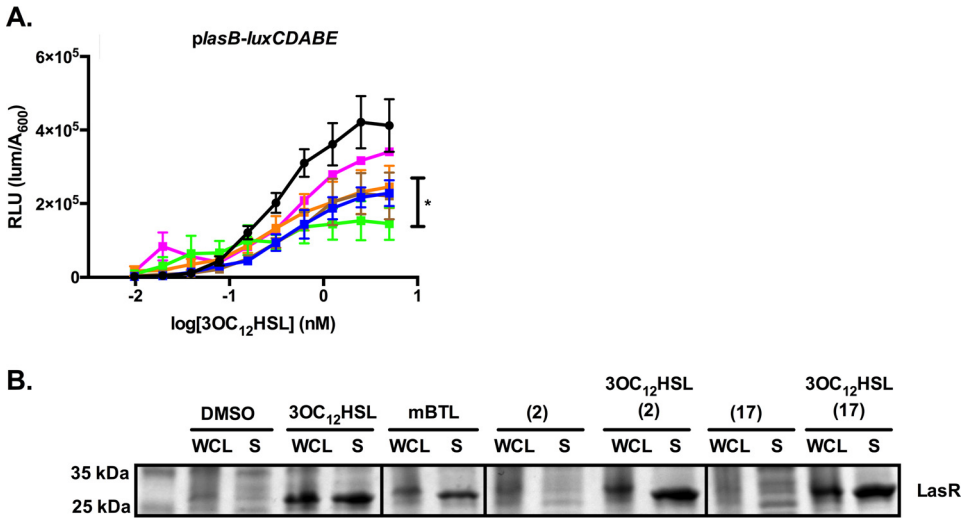


FIGURE 4. **Flavonoids inhibit LasR through a non-competitive mechanism.** *A*, light production from the *E. coli* strain carrying LasR and p*lasB-luxCDABE* in response to $3OC_{12}HSL$ at the specified concentrations in the presence of a 100 $\mu$M concentration of phloretin (**1**) (*blue*), chrysin (**2**) (*brown*), baicalein (**9**) (*light green*), quercetin (**11**) (*orange*), or 7,8-dihydroxyflavone (**17**) (*magenta*). The *black line* shows the response to AI when no flavonoid is present. *Error bars*, S.E. Statistical significance was determined using an ANOVA test with Tukey-Kramer post hoc analysis. The comparison is between the highest concentration of AI supplied and the DMSO-only control. *, $p < 0.04$. *B*, comparison of the whole cell lysates (*WCL*) and supernatants (*S*) from pelleted *E. coli* cells overexpressing LasR under the following conditions: 1% DMSO, or 100 $\mu$M $3OC_{12}HSL$, mBTL, chrysin (**2**), or 7,8-dihydroxyflavone (**17**), as designated.

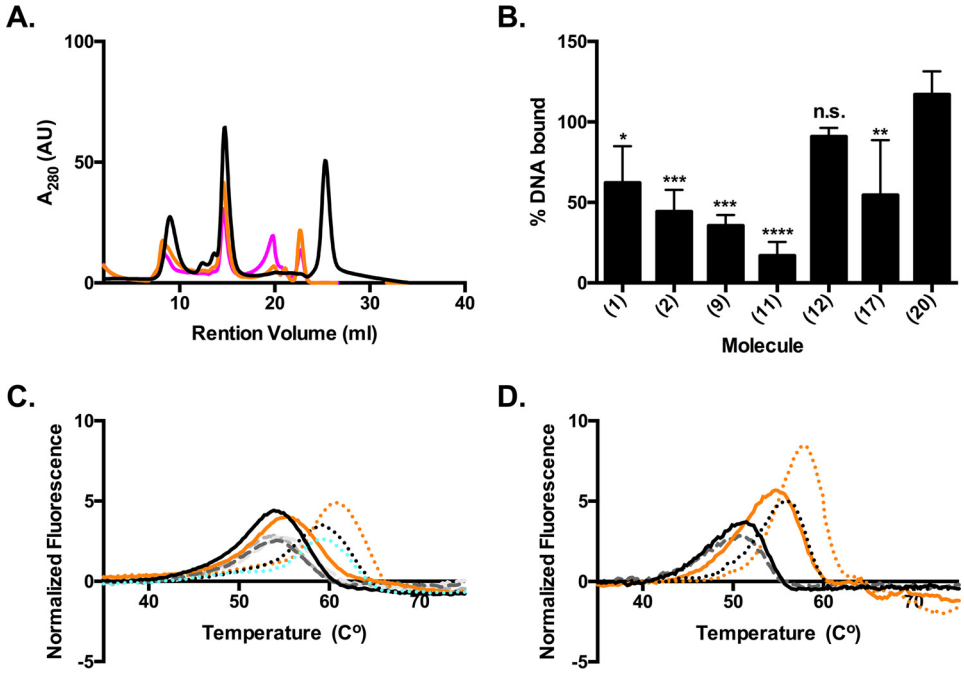


FIGURE 5. **The flavonoids inhibit LasR binding to DNA.** *A*, gel filtration data for full-length LasR bound to $3OC_{12}HSL$ (*black*), to $3OC_{12}HSL$ and quercetin (**11**) (*orange*), and to $3OC_{12}HSL$ and 7,8-dihydroxyflavone (**17**) (*magenta*). Protein was measured by $A_{280}$ (*y* axis) as a function of retention volume (*x* axis). The major LasR species in the protein preparation had a retention volume of 14.6 ml, corresponding to ~53 kDa. A LasR dimer has a molecular mass of 53.2 kDa. *AU* denotes arbitrary units. *B*, quantitation of gel mobility shift assays with LasR-$3OC_{12}HSL$ in combination with 100 $\mu$M phloretin (**1**), chrysin (**2**), baicalein (**9**), quercetin (**11**), 5,7-dimethoxyflavone (**12**), 7,8-dihydroxyflavone (**17**), and baicalin (**20**). 100% DNA binding denotes LasR-$3OC_{12}HSL$ in the absence of any flavonoid. The percentage of DNA bound is the ratio of LasR bound DNA to unbound DNA normalized to the control in the absence of flavonoid. See supplemental Fig. 2 for full data. Statistical significance was determined using an ANOVA test with Tukey-Kramer post hoc analysis. The comparison is the negative control baicalin (**20**). ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. The highest concentration of LasR examined was used for quantification (see supplemental Fig. 2). *n.s.*, not significant. *C*, thermal shift analyses of LasR-$3OC_{12}HSL$ without (*black solid line*) and with an additional 100 $\mu$M $3OC_{12}HSL$ (*black dotted line*), quercetin (**11**) (orange *solid line*), $C_4HSL$ (*silver dashed line*), DPD (*dark gray dashed line*), baicalin (**20**) (*light gray dashed line*), mBTL + $3OC_{12}HSL$ (*cyan dotted line*), and quercetin (**11**) + $3OC_{12}HSL$ (*orange dotted line*). Each *line* represents the average of six replicates. See Table 1 for $\Delta T$ values. *D*, as in *C* for a subset of the molecules under study with the LasR LBD. $\Delta T$ values for additional flavonoids are provided in Table 1.

that we demonstrated in Fig. 1*A* is sufficient for binding by LasR and activation of transcription. LasR-$3OC_{12}HSL$ bound to the *lasB* promoter with high affinity, causing the DNA probe to shift (supplemental Fig. 2) (35). Incubation of LasR-$3OC_{12}HSL$ with a 100 $\mu$M concentration of the test flavonoids phloretin (**1**), chrysin (**2**), baicalein (**9**), quercetin (**11**), and 7,8-diyhydroxyflavone (**17**) prevented LasR-$3OC_{12}HSL$ DNA binding by ~50% or more (Fig. 5*B* and supplemental Fig. 2). Inhibitor

**TABLE 1**
**Flavonoids bind to the full-length LasR and LasR LBD**

| Protein and molecule | Melting temperature[a] | $\Delta T$[a] |
|---|---|---|
| | ℃ | ℃ |
| **LasR** | | |
| DMSO | 53.959 | 0 |
| $3OC_{12}HSL$ | 59.393 | 5.434 |
| $C_4HSL$ | 53.858 | −0.101 |
| DPD | 54.122 | 0.163 |
| **1** | 55.014 | 1.055 |
| **11** | 55.439 | 1.48 |
| **17** | 55.277 | 1.318 |
| **20** | 54.122 | 0.163 |
| mBTL | 60.71 | 6.751 |
| $3OC_{12}HSL$ + **1** | 60.211 | 6.252 |
| $3OC_{12}HSL$ + **11** | 60.447 | 6.488 |
| $3OC_{12}HSL$ + **17** | 60.711 | 6.752 |
| $3OC_{12}HSL$ + mBTL | 59.129 | 5.17 |
| **LasR LBD** | | |
| DMSO | 51.486 | 0 |
| $3OC_{12}HSL$ | 55.967 | 4.481 |
| $C_4HSL$ | 51.75 | 0.264 |
| **11** | 54.649 | 3.163 |
| $3OC_{12}HSL$ + **11** | 58.075 | 6.589 |

[a] The melting temperature (°C) was averaged from six replicates, and the $\Delta T$ (°C) was calculated using the melting temperature of the DMSO control for both full-length LasR and the LasR LBD.

activity in the *lasB-luxCDABE* reporter assay correlated well with the ability of each compound to prevent LasR-$3OC_{12}HSL$ from binding to DNA. Consistent with these data, flavonoids that did not inhibit LasR-dependent transcription in the luciferase reporter assay likewise did not affect LasR-$3OC_{12}HSL$ DNA binding (Fig. 5*B* and supplemental Fig. 2; see 5,7-dimethoxyflavone (**12**) and baicalin (**20**)).

To ensure that the flavonoids were not binding to the DNA substrate itself and in so doing preventing LasR-$3OC_{12}HSL$ from binding, we used thermal shift assays to assess flavonoid binding to LasR-$3OC_{12}HSL$. We incubated 5 $\mu$M LasR-$3OC_{12}HSL$ with 1% DMSO, 100 $\mu$M phloretin (**1**), quercetin (**11**), 7,8-dihydroxyflavone (**17**), baicalin (**20**), $C_4HSL$, or 4,5-dihydroxy-2,3-pentanedione (DPD; an AI made by a variety of bacteria that is commonly called AI-2). The final three molecules, baicalin (**20**), $C_4HSL$, and DPD, represent non-binding control molecules. We melted the protein with a temperature gradient of 0.05 °C/s from 25 to 99 °C. No shift in $\Delta T$ occurred upon the addition of the DMSO control solvent. A large shift in the $\Delta T$ (5.434 °C) (Fig. 5*C* and Table 1) occurred when $3OC_{12}HSL$ was added to LasR-$3OC_{12}HSL$, indicating that as LasR unfolds and releases prebound $3OC_{12}HSL$, additional AI can bind to and restabilize LasR. The addition of $C_4HSL$, DPD, or baicalin (**20**) did not result in a shift in $\Delta T$ (Fig. 5*C* and Table 1). By contrast, phloretin (**1**), quercetin (**11**), and 7,8-dihydroxyflavone (**17**) caused intermediate thermal shifts with $\Delta T$ values of 1.055, 1.480, and 1.318 °C, respectively, (Fig. 5*C* shows quercetin (**11**), and Table 1 provides the data for all test compounds). These results show that the active flavonoids bind directly to the LasR protein. We conclude that flavonoids act as inhibitors via mechanism 4, by preventing LasR from binding to promoter DNA.

We did not test mechanism 5, impairment of the ability of LasR to interact with RNA polymerase, because our findings show that DNA binding, the step in the LasR-transcriptional activation process preceding LasR-RNA polymerase engagement, is disrupted by the flavonoids.

*Flavonoids Can Bind in the LasR Ligand Binding Domain When AI Is Bound*—To pinpoint which LasR domain is bound by the flavonoid inhibitors, we performed thermal shift analyses with the purified LasR ligand binding domain (LBD) bound to $3OC_{12}HSL$ and two of our test compounds. The exogenous addition of $3OC_{12}HSL$ or quercetin (**11**) caused thermal shifts similar to those obtained when each compound was added to full-length LasR-$3OC_{12}HSL$, indicating that the flavonoids bind to the LBD (Fig. 5*D* and Table 1). Consistent with our above results, we did not observe a significant thermal shift when $C_4HSL$ or baicalin (**20**) was added to the $3OC_{12}HSL$-bound LasR LBD (Table 1). We were unable to similarly assay the LasR DNA-binding domain due to insufficient protein yields. Thus, we conclude that the inhibitors under study bind the LasR LBD and non-competitively prevent DNA binding.

We used thermal shift analyses in which we added combinations of AI and flavonoid inhibitors to discern whether or not the flavonoids, while acting non-competitively, nonetheless employ the LasR canonical ligand binding site. Our rationale is that if the flavonoids bind in the LasR ligand binding pocket, when both flavonoid and AI are added to LasR-$3OC_{12}HSL$, either the flavonoid or the AI would exchange with prebound AI, resulting in a thermal shift that is a consequence of one molecule binding. If, on the other hand, the flavonoids use a discrete binding site, AI from solution could exchange with prebound AI and flavonoid could simultaneously bind elsewhere on LasR, resulting in a cumulative thermal shift that is a consequence of two molecules binding. In Fig. 5*C* and Table 1 and as mentioned, we show that exchange of soluble AI with bound AI at the LasR ligand binding site causes a $\Delta T$ of 5.434 °C. In agreement with this result, the addition of mBTL, the known competitive inhibitor of LasR, in combination with $3OC_{12}HSL$, causes a similar change ($\Delta T$ = 5.170 °C; Fig. 5*C* and Table 1), showing that the effect is not cumulative. When we carried out the analysis with phloretin (**1**), quercetin (**11**), and 7,8-dihydroxyflavone (**17**) together with $3OC_{12}HSL$, increases in the $\Delta T$ to 6.252, 6.488, and 6.752 °C occurred, respectively (Fig. 5*C* and Table 1), suggesting that the AI and the flavonoid inhibitors can simultaneously bind to LasR. We interpret this result to mean that the flavonoids do not use the canonical AI binding site.

Interestingly, the thermal shift with mBTL alone ($\Delta T$ = 6.751 °C) was higher than that for mBTL with $3OC_{12}HSL$ or for $3OC_{12}HSL$ alone (Table 1). We attribute this result to the ability of mBTL to act as a LasR agonist at low concentrations (18). Specifically, the $EC_{50}$ for mBTL to activate LasR is 9.3 nM compared with 2.1 nM for $3OC_{12}HSL$ (supplemental Fig. 3, *A* and *B*). The higher $EC_{50}$ for mBTL is probably due to a non-diffusion-limited association rate. mBTL more efficiently stabilizes QS receptors than do native AIs (18), suggesting that the dissociation rate of mBTL from receptor is slower than that of AI. This increased binding energy for mBTL relative to AI possibly results in the larger $\Delta T$ for mBTL. When both mBTL and $3OC_{12}HSL$ are added to LasR simultaneously, due to the lower $EC_{50}$, the AI outcompetes mBTL for the ligand binding site, and we observe a shift similar to that of AI alone ($\Delta T$ = 5.17 °C for

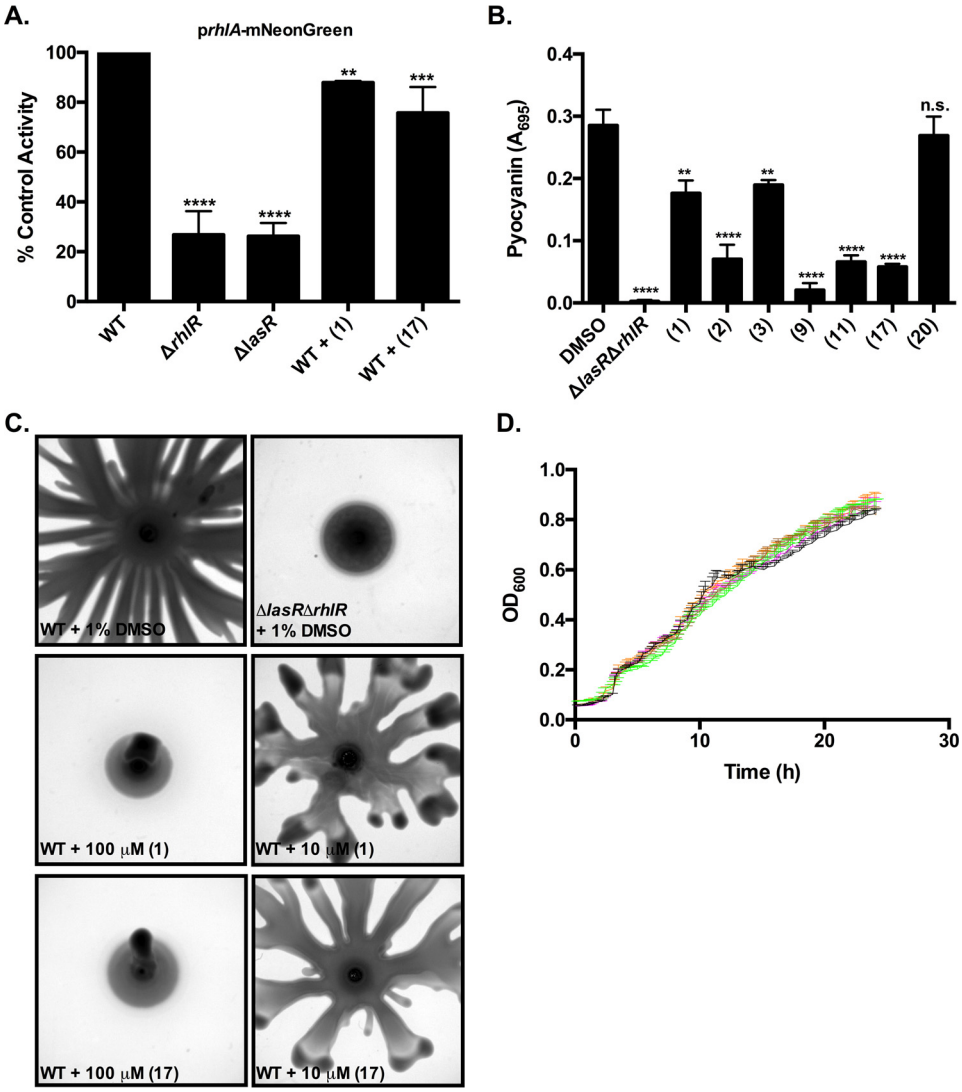


FIGURE 6. **Flavonoids inhibit transcription of *rhlA*, reduce pyocyanin production, and suppress swarming in *P. aeruginosa*.** *A*, the *rhlA* promoter sequence was transcriptionally fused to mNG on the wild-type *P. aeruginosa* PA14, Δ*lasR*, and Δ*rhlR* chromosomes. All strains were grown to $A_{600} = 2.0$, and the response to 100 μM phloretin (**1**) and to 100 μM 7,8-dihydroxyflavone (**17**) was monitored by measuring fluorescence. $n = 3$. Statistical significance was determined using an ANOVA test with Tukey-Kramer post hoc analysis. The comparison is between wild-type and the DMSO control. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$. *B*, pyocyanin production is shown for wild-type *P. aeruginosa* PA14 in the presence of DMSO and a 100 μM concentration of the designated flavonoids. The Δ*lasR*Δ*rhlR* double mutant is the negative control. $n = 3$. Statistical significance was determined using an ANOVA test with Tukey-Kramer post hoc analysis comparing the wild type with the DMSO control. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$. *n.s.*, not significant. *C*, wild-type *P. aeruginosa* PA14 (designated WT) and the Δ*lasR*Δ*rhlR* double mutant swarming phenotypes on soft agar medium. WT swarming is also shown in the presence of 1% DMSO and 100 and 10 μM phloretin (**1**) or 7,8-dihydroxyflavone (**17**). *D*, growth of wild-type *P. aeruginosa* PA14 in the absence (DMSO; *black*) or presence of 100 μM chrysin (**2**) (*brown*), baicalein (**9**) (*light green*), quercetin (**11**) (*orange*), and 7,8-dihydroxyflavone (**17**) (*magenta*) in LB medium over 24 h. $n = 3$. *Error bars*, S.E.

AI and mBTL together, $\Delta T = 5.434$ for AI alone) (Table 1). The differing $\Delta T$ values for the competitive compound mBTL and the flavonoids reinforce our conclusion that flavonoids do not use the AI binding site.

*Flavonoids Inhibit QS-dependent Transcription and QS-directed Behaviors in Vivo*—The above results show that particular flavonoids inhibit LasR and RhlR in recombinant *E. coli*, and they inhibit purified LasR protein *in vitro*. To examine the *in vivo* consequences of flavonoid inhibition of QS receptors, we first assayed regulation of the QS-controlled promoter *rhlA* fused to mNeonGreen (mNG) in *P. aeruginosa* PA14. We confirmed that our chromosomal reporter, P*rhlA*-mNG, was QS-regulated by assaying its expression in different QS mutant backgrounds. As expected, compared with wild-type *P. aeruginosa* PA14, the Δ*lasR* and Δ*rhlR* mutants exhibited reduced P*rhlA*-mNG expression (Fig. 6*A*). The addition of 100 μM phloretin (**1**) or 7,8-dihydroxyflavone (**17**) to wild-type *P. aeruginosa* caused 15 and 24% decreases, respectively, of P*rhlA*-mNG expression. These results indicate that both molecules function *in vivo* to inhibit QS-controlled transcription of a target gene. Only modest inhibition is expected in this assay because complete loss of *rhlA* expression does not occur in QS mutants (Fig. 6*A*); nor does complete inhibition of *rhlA* expression occur in the *E. coli* reporter assay when phloretin (**1**) or 7,8-dihydroxyflavone (**17**) is provided (Fig. 2*A*).

We also measured production of pyocyanin, a QS-controlled virulence factor. All of the flavonoids that we characterized as LasR/RhlR inhibitors significantly decreased pyocyanin pro-

duction in *P. aeruginosa* PA14 (Fig. 6*B*). Flavonoids that were characterized as inactive in our above tests did not reduce pyocyanin production. As a reference, we show that decreased pyocyanin production occurs in the double Δ*lasR*Δ*rhlR* mutant. Finally, consistent with the pyocyanin and transcriptional assays, supplementation of soft agar with the flavonoid inhibitors suppressed the QS-activated swarming phenotype (Fig. 6*C* shows phloretin (**1**) and 7,8-dihydroxyflavone (**17**)). None of the test molecules altered growth in *P. aeruginosa* PA14 (Fig. 6*D*), indicating that the observed effects stem from the inhibition of QS. Taken together, these findings show that the flavonoid inhibitors function *in vivo* through the QS circuit.

## Discussion

This work demonstrates that flavonoids are inhibitors of the QS receptor LasR and that they do not function via a competitive mechanism involving displacement of the natural AI from its binding pocket. Rather, the flavonoids bind to the LasR LBD and prevent the protein from binding to DNA. RhlR has, for 2 decades, proven intractable to purification and biochemical analysis. We expect our LasR mechanistic interpretations to extend to RhlR, given the similarity between LasR and RhlR; however, we are unable to show this directly. We have shown that the flavonoids inhibit both LasR and RhlR in our recombinant *E. coli* reporter assays. *In vivo*, the flavonoids inhibit transcription of *rhlA*, reduce pyocyanin production, and prevent swarming. A previous report noted that dihydroxyflavonoids repress certain QS behaviors, such as pyocaynin production, elastase production, and biofilm formation in *P. aeruginosa* and other bacteria (20–22). However, the flavonoid mechanism of action was not studied before the present work. Our findings underpin those earlier results with mechanistic insight.

Our findings are also consistent with previous results showing that flavonoids can bind to particular transcription factors that contain variable LBDs and canonical helix-turn-helix DNA-binding domains. One example is the TetR-type transcription factor, TtgR. The crystal structure of TtgR bound to quercetin (**11**) and narigenin (**3**) revealed that binding relied on six amino acids forming hydrogen bonds with the hydroxyl groups on the quercetin (**11**) and narigenin (**3**) rings. The structure of TtgR bound to phloretin (**1**) showed that phloretin (**1**) uses a binding pocket similar to that used by narigenin (**3**) and quercetin (**11**) as well as a second, adjacent binding pocket (37). However, phloretin (**1**) could not occupy both binding sites in the same monomer. The binding of these flavonoids to TtgR disrupted the ability of TtgR to bind to DNA, similar to our findings with LasR (37). Simulations of flavonoids binding to other transcription factors indicate that $\pi$-stacking interactions could stabilize flavonoid-protein interactions (38, 39). For example, the intermolecular forces stabilizing the interaction between nsP3 (non-structural protein 3) from the Chikungunya virus and baicalein (**9**) were calculated, and extensive hydrogen bonding with the hydroxyl groups in the A-ring of baicalein (**9**) was predicted. An important $\pi$-$\pi$ interaction was also predicted to occur between a tryptophan residue on nsP3 and the B-ring of baicalein (**9**) (39). Consistent with these data suggesting that flavonoid ligands employ multiple amino acids for binding, we have been unable to identify any single missense mutation in LasR that confers resistance to the flavonoids.

All LasR and RhlR inhibitors reported previously function by binding in the ligand binding site of the receptor LBD (18, 40). Our understanding of the mechanistic consequences of such competitive inhibitory mechanisms in this protein family was accelerated by the solution of the structure of CviR from *Chromobacterium violaceum* bound to the competitive inhibitor CL (27). CL, when in the AI binding site, induces a crossed domain, closed conformation that locks the CviR DNA binding helices into a configuration that is incompatible with DNA binding (27). Although not competitive, the flavonoids also prevent DNA binding. It is possible that by binding in the LBD, the flavonoids cause long range conformational changes that also lock the DNA-binding domain into an unfavorable configuration.

Flavonoids may present an exciting avenue for future pharmacological development, given that the work described here provides a new mechanism of action for them. For example, approaches similar to those employed here could be used to explore flavonoid inhibition of QS systems in other pathogenic bacteria (21). With respect to *P. aeruginosa*, potential applications of the present findings are enhanced by a recent report showing that QS activates CRISPR-Cas (clustered regularly interspaced short palindromic repeats) adaptive immunity in *P. aeruginosa* PA14 (41). *P. aeruginosa* PA14 uses the CRISPR-Cas system to eliminate invading phages. QS activates CRISPR-*cas* expression, increases activity, and enhances adaptation, presumably optimizing the timing and level of deployment of this defensive mechanism. Inhibition of QS through small molecules, such as flavonoids, could effectively repress virulence factor production while simultaneously rendering *P. aeruginosa* more susceptible to phage infection through suppression of QS-directed activation of CRISPR-Cas immunity (41). Phage therapy is not widely used in the United States; however, it is being revisited in light of current developments in antibiotic resistance, and it is an accepted antimicrobial therapy in other nations. We suggest that phage therapy coupled to QS inhibition in *P. aeruginosa* could be explored as a combination therapy with far reaching implications, perhaps beyond medicine, for animal husbandry, agriculture, and engineering.

Flavonoids are produced by plants as secondary metabolites, and they have a range of pharmacological effects (19, 42). For instance, chrysin (**2**), described here as an inhibitor of LasR and RhlR, is an inhibitor of glycogen phosphorylase and is proposed to have the potential to control hyperglycemia in type 2 diabetes patients (43). Other flavonoids possess traditional antibiotic activity (44, 45). Flavonoids combined with conventional antibiotics can enhance the efficacy of the antibiotic, as is the case with 6,7-dihydroxyflavone and $\beta$-lactam antibiotics in methicillin-resistant *Staphylococcus aureus* (46). Thus, exploration of flavonoids, either alone or in combination with existing therapies, for new uses, such as we have shown here for QS, seems a promising route.

In their native roles as plant metabolites, flavonoids are crucial for root nodule development (47). Specifically, some actinorhizal plants require nitrogen-fixing rhizobial symbionts. In this relationship, the rhizobia convert atmospheric nitrogen

($N_2$) to a usable form for the plant ($NH_3$), whereas the plant, in turn, provides sugars as carbon sources for the bacteria (47). Flavonoids activate *nod* gene expression in rhizobia, and the bacterial Nod components are required for nodule development in the plant, establishing the location for nitrogen fixation (48, 49). Flavonoids can also repress bacterial *nod* gene expression, allowing the plant and bacteria to fine tune their interactions such that overexpression of *nod* factors does not occur, which avoids initiation of the host plant's defense response (45). One flavonoid, narignenin (**3**), induces *nod* gene expression in rhizobia, and here we show that it is also a potent LasR and RhlR inhibitor (50). Given that we and others have shown that flavonoids function as transcription factor modulators, we speculate that flavonoids could have evolved as a consequence of the plants' need to influence transcriptional regulation in bacterial symbionts and, perhaps, in pathogens as well. Indeed, *P. aeruginosa* is a ubiquitous bacterium, and it is found in the rhizosphere, where it acts as a pathogen that relies on QS for virulence (51, 52).

Flavonoid production in plants and AI signaling in bacteria have other known links because certain legumes produce flavonoids in response to the presence of bacterial AIs (53). Additionally, in a few instances, plants produce QS mimics in response to bacterial cues. For instance, *p*-coumaric acid is exuded by legume roots, and this molecule can alter QS signaling in bacteria that use *p*-coumaroyl HSL as an AI (54, 55). It is particularly fascinating to envision instances of co-evolution in which plants produce flavonoids that function to maintain symbiotic bacteria via enhancement of QS while simultaneously suppressing potential pathogens through inhibition of QS (56). Our findings indicate that such natural products have promise as plausible alternatives/supplements to traditional antibiotics and as possible new stand-alone medicines (24).

## Experimental Procedures

*P. aeruginosa Strain Construction*—To construct the P*rhlA*-mNeonGreen transcriptional reporter, 500 bp of DNA upstream of the *rhlA* gene and the mNeonGreen open reading frame were amplified using *P. aeruginosa* PA14 genomic DNA and the plasmid pmNeonGreen-N1 (licensed from Allele Biotech) (57) as templates, respectively. Next, two DNA fragments of ~730 bp, one corresponding to the intergenic region ~700 bp downstream of the PA14_20500 gene and the other corresponding to ~1000 bp upstream of PA14_20510, were amplified using PA14 genomic DNA as templates. The four DNA fragments were stitched together by Gibson assembly and cloned into pEXG2 (58, 59). The resulting plasmids were used to transform *E. coli* SM10 *λpir* and then mobilized into *P. aeruginosa* via biparental mating. Transconjugants were selected on LB agar containing gentamicin (30 μg/ml) and irgasan (100 μg/ml), followed by recovery of mutants on LB agar plates containing 5% sucrose. Candidate integration mutants were confirmed by PCR.

*LasR/RhlR lux Reporter Assay*—The *lasR* and *rhlR* genes were cloned into the pBAD-A expression vector using standard molecular biological techniques. The transcriptional reporters were generated by PCR amplification of the promoters (−20 to −250) of *P. aeruginosa* PA14 *lasB* and *rhlA* and cloning them upstream of the *luxCDABE* operon from *V. harveyi* (pCS26). A ribosome binding site sequence was inserted to enhance translation of the luciferase mRNA. Plasmids were co-transformed into TOP10 *E. coli* cells (Invitrogen) and plated on LB containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). Three colonies were selected and grown overnight in 3 ml of LB at 37 °C. Cultures were back-diluted 1:1000 in fresh LB medium containing antibiotics and grown at 37 °C until $A_{600} = 0.5$, or for ~4 h. For inhibition assays, arabinose was added to a final concentration of 0.1%. AIs were used at 2.5 nM and 10 μM for LasR and RhlR, respectively. For dose-response assays, 100× stocks were generated in DMSO. These stocks were assayed at various dilutions in the reporter assays, starting at 100 μM. Assays were carried out in 96-well plates (Corning). Plates were incubated at 30 °C for 4 h, and bioluminescence was measured on an Envision 2103 Multilabel Reader (PerkinElmer Life Sciences) with a measurement time of 0.1 s. $A_{600}$ was measured using a photometric 600-nm filter at 100% light emission. Relative light units (RLU) were calculated by dividing the bioluminescence measurement by the $A_{600}$ measurement. To construct the constitutively bioluminescent reporter strain, the DNA encoding the *tac* promoter was amplified and cloned upstream of the *luxCDABE* operon from *V. harveyi* to make plasmid pCS26. pCS26 was transformed into TOP10 *E. coli*, which does not possess the *lacIQ* genes, resulting in constitutive expression of *luxCDABE*. For consistency with our primary screen, the *lasR*-containing plasmid pJP100 was transformed into this strain. Dose-response assays were performed on hit compounds as described above. Compounds that reduced light production in this strain were eliminated from further analyses. The CviR and LuxN reporter assays were performed as described previously (28).

*High Throughput Small Molecule Screen*—The plasmids carrying *lasR* and *lasB-luxCDABE* were co-transformed into TOP10 *E. coli* cells (Invitrogen) and grown as described above. Stationary phase cultures were back-diluted 1:1000 in 250 ml of fresh LB medium containing antibiotics and grown at 37 °C until $A_{600} = 0.5$ or for ~4 h with shaking. Arabinose was added to a final concentration of 0.1% (v/v), and AI was added to 2.5 nM. 384-well plates (Corning) were supplied with 200 nl of 10 mM stock compounds dissolved in DMSO from the Princeton University Small Molecule Screening Center. 20 μl of the reporter strain culture was next added by an automatic plate filler (Thermo Multidrop Combi). The first two and final two columns of each plate were left blank as controls. Positive control wells received arabinose, AI, and DMSO but no small molecule candidate. Negative control wells received no AI. Plates were incubated at 30 °C for 4 h, bioluminescence and $A_{600}$ were measured, and RLU were calculated as described above. The average and S.D. were calculated for the positive control group. For each plate, the $Z'$ was calculated, and hits were only considered for wells with $Z' > 0.7$. Compounds that caused bioluminescence reductions at least three S.D. values from the mean were considered hits and were retested from frozen stocks. Once confirmed, candidate hit compounds were obtained as powders (Sigma and Cayman Chemical) and retested.

*LasR Protein Purification*—Full-length LasR (cloned into pET23b) was produced in BL21 *E. coli* cells using 1 mM IPTG at

18 °C overnight in the presence of 100 μM $3OC_{12}$HSL. Cells were pelleted at 3000 rpm and resuspended in lysis buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8, 20 mM imidazole, 1 mM EDTA, 1 mM DTT, 5% glycerol). The cell resuspension was lysed using sonication (1-s pulses for 15 s with a 50% duty cycle). The soluble fraction was isolated using centrifugation at 32,000 × *g*. To prepare the protein for heparin column binding, the soluble fraction was diluted 5-fold in buffer A (20 mM Tris-HCl, pH 8, 1 mM DTT). The protein was loaded on to a heparin column (GE Healthcare) and eluted using a linear gradient from buffer A to buffer B (1 M NaCl, 20 mM Tris-HCl, pH 8). Peak fractions were collected and assessed by SDS-PAGE analysis. Fractions were pooled and again diluted 5-fold in buffer A and then loaded onto a MonoQ column (GE Healthcare) and eluted using a linear gradient from buffer A to buffer B. Peak fractions were collected for SDS-PAGE analysis, pooled, and concentrated for size exclusion chromatography on a GE Healthcare S200 column in 20% buffer B. Peak fractions were pooled, concentrated to 2 mg/ml, flash-frozen, and stored at −80 °C. $His_6$-tagged LasR LBD was produced as described for the full-length protein. The soluble fraction was applied to a nickel-nitrilotriacetic acid column and eluted using a linear gradient of buffer C (200 mM NaCl, 20 mM Tris-HCl, pH 8, 20 mM imidazole, 1 mM DTT) to buffer D (200 mM NaCl, 20 mM Tris-HCl, pH 8, 1 M imidazole, 1 mM DTT). Peak fractions were pooled, and protein homogeneity was verified on a S200 size exclusion gel filtration column as described above.

*Electrophoretic Mobility Shift Assay*—The *lasB* promoter sequence (−250 to −20) was amplified using PCR and end-labeled with $^{32}P$ using PNK enzyme (Fermentas). The labeled probe was incubated with 0, 25, and 50 ng of purified LasR-$3OC_{12}$HSL in binding buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 50 mM KCl, 1.5 mg/ml poly(I-C), 50 μg/ml BSA, and 10% glycerol) containing DMSO or 100 μM flavonoid in DMSO for 30 min at 37 °C. DNA-protein complexes were subjected to electrophoresis on 6% native polyacrylamide gels and visualized using a Typhoon PhosphorImager (GE Healthcare).

*Thermal Shift Assay*—LasR-$3OC_{12}$HSL protein was diluted to ~5 μM (based on $A_{280}$ measurement) in reaction buffer (20 mM Tris-HCl, pH 8, 200 mM NaCl, and 1 mM DTT) containing DMSO or 100 μM flavonoid, AI, or mBTL in DMSO in an 18-μl total volume and allowed to incubate at room temperature for 15 min. 5000× SYPRO Orange in DMSO was diluted to 200× in reaction buffer and used at 20× final concentration (2 μl added to each 18-μl reaction). 20-μl samples were assessed for thermal shifts in 384-well plates on a Quant Studio 6 Flex System (Applied Biosystems) using the melting curve setting and measuring fluorescence using the ROX reporter setting. Samples were incubated at 25 °C for 2 min and then subjected to a linear gradient of 0.05 °C/s until the temperature reached 99 °C, where it was held constant for 2 min.

*P. aeruginosa rhlA Reporter Assay*—Wild-type and mutant *P. aeruginosa* PA14 strains harboring the P*rhlA*-mNG fusion were grown overnight and diluted 1:1000 in 3 ml of LB medium. DMSO solvent, phloretin (**1**), or 7,8-dihydroxyflavone (**17**) was added at 100 μM, and the cultures were incubated at 37 °C for 5 h. The cultures were subjected to centrifugation at 3000 rpm, and the cells were resuspended in fresh LB medium containing DMSO, 100 μM phloretin (**1**), or 100 μM 7,8-dihydroxyflavone (**17**). The resuspended cells were allowed to grow for another 3 h. 1 ml of culture was harvested, and the cells were pelleted at 3000 rpm. The supernatant was removed, and the cells were resuspended in PBS. 200 μl of cells were transferred to a 96-well plate, and fluorescence was measured using an Envision 2103 multilabel reader (PerkinElmer Life Sciences) using the FITC filter with an excitation of 485 nM and emission of 535 nM.

*Pyocyanin Assay*—Wild-type *P. aeruginosa* PA14 was grown overnight in LB liquid medium at 37 °C with shaking in the presence of DMSO or 100 μM mBTL or test flavonoid. Cultures were back-diluted 1:1000 into fresh medium containing appropriate test compounds. The cultures were grown for 5 h and back-diluted 1:50 into fresh medium containing test compounds. The cultures were grown for 18 h. The cells were pelleted by centrifugation, and the cell-free culture fluids were passed through 0.22-μm filters into clear plastic cuvettes. The $A_{695}$ of the filtered fluids was measured on a spectrophotometer (Beckman Coulter DV 730).

*Swarming Assay*—Cultures of *P. aeruginosa* PA14 and the Δ*lasR*Δ*rhlR* mutant were grown overnight in LB with DMSO, 100 μM phloretin (**1**), or 100 μM 7,8-dihydroxyflavone (**17**). 2 μl of the stationary phase cultures were spotted onto swarming agar medium (Luria-Bertani broth (Thermo) with 0.5% (w/v) casamino acids, 0.5 (w/v) glucose, and 0.5% Bacto agar) that had been supplemented with 1% DMSO or with 10 μM or 100 μM phloretin (**1**) or with 10 μM or 100 μM 7,8-dihydroxyflavone (**17**). The plates were incubated overnight at 37 °C and imaged after 24 h using an Image Quant LAS4000 gel dock using the trans-illumination setting (GE Healthcare).

*Growth Curve*—Cultures of *P. aeruginosa* PA14 were grown overnight in LB medium with 1% DMSO or 100 μM test flavonoids, back-diluted 1:1000 into LB containing DMSO or a 100 μM concentration of the same flavonoid, and transferred to a 96-well plate. The plate was incubated in a BioTek Eon plate reader overnight with shaking at 37 °C. $A_{600}$ was measured every 15 min.

*Author Contributions*—J. E. P. conceived and coordinated the study; wrote the paper; and designed, performed, and analyzed the experiments shown in Figs. 1–6. S. M. provided technical assistance, constructed mutants, and performed experiments shown in Fig. 6. A. R. M. provided technical assistance and contributed to the preparation of the figures. J.-P. C. provided technical assistance and performed the experiments in Figs. 1 and 2. B. R. H., C. J. A., H. K., and C. D. S. assisted in the conception and coordination of the study and provided technical assistance. H. K., B. R. H., and C. D. S. provided important intellectual input. B. L. B. conceived and coordinated the study, analyzed data, and wrote the paper.

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